STANLEY, JAMES LEE. Serological Typing and Chlorine Resistance of Cyanophages Isolated From Wastewater. (1976) Directed by: Dr. Robert E. Cannon. Pp. 50.

No viral monitoring systems currently exist in our wastewater treatment facilities. The assays are impractical in treatment plant laboratories because of their cost and complexity. In addition it is difficult to isolate animal viruses from large volumes of water. However, cyanophages, the virus of blue-green algae, can serve as indicators of the presence of animal viruses. Cyanophages are ubiquitous in wastewater throughout the year. Both cyanophage LPP-1 and LPP-2 are more resistant to chlorine disinfection than are coliform bacteria and enteric animal viruses from human feces. The detection and assaying materials for cyanophages are reliable, inexpensive, and practical for the laboratories of wastewater reclamation facilities. By the utilization of cyanophages as pollution indicators; viral and bacterial, a more reliable method is available to monitor the disinfection processes in sewage treatment.

SEROLOGICAL TYPING AND CHLORINE RESISTANCE OF CYANOPHAGES ISOLATED FROM WASTEWATER

by

James Lee Stanley

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Arts

Greensboro
1976

Approved by

Robert E Cameron
Thesis Advisor

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

Thesis Adviser Robert E Cameron

Oral Examination Committee Members Robert E Cameron

Sarah Sands

Richard Morse

June 30, 1976
Date of Examination

ACKNOWLEDGEMENTS

Special thanks to Dr. Robert E. Cannon for his support and guidance of this study.

Appreciations go to Bill Smith, Ken Brown and Dan Holder of the North Buffalo Creek Sewage Treatment Plant, to Miss Sarah Sands and Dr. Richard Morse for their assistance with this project. Also thanks to Miss Nancy Elliott for statistical counceling.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Because of supply and demand, it is mandatory that water be used repeatedly. The demand for usable water is greater than available supplies. Presently, wastewater treatment plants are relied upon to reclaim the water. Methods are available to purge wastewater of all hazardous materials to ensure purity, but there is a disparity between levels of purity desired and economic allocations. The receiving waters from waste treatment plants usually do not possess high water quality, because economic guidelines rather than sanitary guidelines determine the degree of water quality.

DOMESTIC SEWAGE

Sewage plants that process domestic wastewater are required to perform tests on the wastewater to monitor the efficiency of the purification processes. Treatment facilities, with certain variations depending on their classification, perform the required tests and measurements listed in table 1 (State of North Carolina Department of Natural and Economic Resources, 1975).

TABLE 1

Required Tests and Measurements	Location of Sampling Points
Settleable Matter	Effluent
Temperature	Effluent, Up & Downstream
Dissolved Oxygen	Up & Downstream
Residual Chlorine	Effluent
pH	Influent, Effluent, Up & Downstream
BOD*	Influent, Effluent, Up & Downstream
COD+	Effluent, Up & Downstream
Coliform Fecal	Effluent, Up & Downstream
Total Residue	Influent, Effluent
Total Suspended Residue	Influent, Effluent
Total Kjeldahl Nitrogen	Influent, Effluent

*BOD= Biochemical Oxygen Demand.
+COD= Chemical Oxygen Demand.

Bacteriological, physical, and chemical characteristics of wastewater can be analyzed and attempts can be made to maintain each at acceptable levels. Fecal coliforms serve as indicators of bacterial pollution. If coliforms are found in significant numbers, then there is a possibility that bacterial pathogens may be present, such as Salmonella, Shigella, or Vibrio cholera. The presence of coliforms in water is determined either by the Most Probable Number (MPN) or Membrane Filtration (MF) methods, as described in Standard Methods (1965). The MPN index should not exceed 10 coliforms per 100 ml or there should be no more than 4 coliforms per 100 ml by the MF method (Zajic, 1971).

Physical characteristics are also considered when determining water quality. Though bacterial characteristics are more important for health reasons, certain physical criteria have been established for treated wastewater as shown in table 2 (Zajic, 1971).

TABLE 2

Physical Quality of Treated Water

Parameter	Objective	Acceptable Limit
Color TCU_1	5 mg/L	15 mg/L
Odor TON_2	0	4 mg/L
Taste	Inoffensive	Inoffensive
Turbidity JTU_3	1 mg/L	5 mg/L
Temperature	10°C	15°C
pH	___	6.5 - 8.3

1TCU= True Color Unit.
2TON= Threshold Odor Unit.
3JTU= Jackson Turbidity Unit.

Numerous toxic or harmful chemicals can and do make their way to water supply. Presently, under normal conditions testing is done twice a year, unless suspected chemical pollution is present (Zajic, 1971). Since chemical pollution is a broad and variable problem, analytical tests have to be performed to detect such chemical groups as cyanides, phenolic compounds, radioactive isotopes, carbonates, sulfates, and other potentially hazardous chemical substances.

SEWAGE TREATMENT

Sewage contains mineral and organic matter in solution, as well as colloidal and coarse suspended matter (Imhoff and Fair, 1956). Water has proved to be a convenient vehicle to carry away domestic and industrial waste. To ensure that this water is safe for reuse, the harmful or offensive constituents must be removed. This may be accomplished by a variety of sewage treatment methods.

Initially, wastewater goes through a preparatory treatment process to separate coarse suspended and floating matter. This is achieved by the following processes (Imhoff and Fair, 1956):

1. Separation of Coarse Suspended and Floating Matter: Screening
   - A. Fine Screens
   - B. Bar Racks
   - C. Cutting Screens or Comminuters
2. Separation of Grease and Oil: Skimming or Flotation
   - A. Separate Skimming Tanks
   - B. Settling Tanks with Scum Collectors

Preparatory treatment is followed by primary treatment which consists of the removal of finely divided suspended matter. The wastewater treatment steps that accomplish this are (Imhoff and Fair, 1956):

3. Separation of Finely Divided Suspended Matter: Sedimentation and Chemical Precipitation
   - A. Grit Chambers and Detritus Tanks
   - B. Settling or Sedimentation Tanks

1. Plain Sedimentation Tanks
2. Chemical Precipitation Tanks
3. Septic Tanks

4. Separation of Finely Divided Suspended Matter: Filtration
   A. Land Treatment or Irrigation
   B. Intermittent Sand Filtration
   C. Rapid Sand or Magnetite Filtration

The third step in sewage treatment involves the removal of solids by the biological or secondary treatment process. Nature has provided means to self-purify its waters, and man has adapted these natural biological degradation principles to purify waste. The microbial oxidation reduction process has been enhanced in the concentrated area of the treatment plant. A substantial reduction of pollutants can be attained by the oxidation process within six to nine hours (James, 1971). This particular segment of sewage treatment can be completed by the ensuing methods (Imhoff and Fair, 1956):

5. Separation or Stabilization of Putrescible Matter in Suspension, the Colloidal State, or Solution: Biological Treatment
   A. Land Treatment or Irrigation
   B. Intermittent Sand Filtration
   C. Trickling Filters
   D. Activated-sludge Units
   E. Oxidation Ponds

Matter separated from wastewater, such as coarse material and sludge from biological treatment, is disposed of in digestion tanks, land fills, or dried and incinerated.

The wastewater load has become so great that the majority of our waste treatment plants presently have a terminal disinfection process to further reduce hazards associated with sewage. Chlorination of wastewater reduces the coliform level significantly, and also improves physical and chemical aspects of the water. Chlorine is added terminally to biologically treated effluents, either in the form of a gas or liquid, to maintain a residual chlorine level between 0.2 to 1.0 mg/L for twenty minutes (Holder, 1976).

Three important parameters used to determine the efficiency of treatment are biochemical oxygen demand (BOD), amount of suspended solids, and bacterial presence. The BOD is a measurement of the amount of dissolved oxygen used by saprophytic organisms during decomposition. Therefore, it can be considered an indirect measurement of the amount of decomposing organic matter (Imhoff and Fair, 1956). Determination of bacteria and suspended solids can be done quantitatively with results providing direct evidence of sewage treatment treatment performance. The relative efficiencies of sewage treatment are shown in table 3 (Imhoff and Fair, 1956).

TABLE 3

Relative Efficiencies of Sewage-treatment Operations or Processes, Percentage Removal

Treatment Operation or Process	BOD	Suspended Solids	Bacteria
1. Fine screening	5 to 10	2 to 20	10 to 20
2. Chlorination of raw or settled sewage	15 to 30	_____	90 to 95
3. Plain sedimentation	25 to 40	40 to 70	25 to 75
4. Chemical precipitation	50 to 85	70 to 90	40 to 80
5. High-rate trickling filtration preceded and followed by plain sedimentation	65 to 95	65 to 92	80 to 95
6. Low-rate trickling filtration preceded and followed by plain sedimentation	80 to 95	70 to 92	90 to 95
7. High-rate activated-sludge treatment preceded and followed by plain sedimentation	65 to 95	65 to 95	80 to 95
8. Conventional activated sludge treatment preceded and followed by plain sedimentation	75 to 95	85 to 95	90 to 98
9. Intermittent sand filtration	90 to 95	85 to 95	95 to 98
10. Chlorination of biologically treated sewage	_____	_____	98 to 99

Percentage removal or efficiency= 100 X (influent concentration — effluent concentration) ÷ influent concentration

CHLORINE AS A DISINFECTANT

Terminal disinfection by chlorination is employed throughout the world in an attempt to protect against waterborne infectious agents. Ninety-five percent of all municipal potable water disinfection is done by chlorination (DeMichele, 1974). In order for chlorine to be effective as a disinfectant specific conditions must be maintained. Chlorine is most effective between the pH range of 5 - 8.5, when no interfering substances are present, such as nitrogen compounds. Chlorine is present mainly as hypochlorous acid (HOCl) between pH 5 - 8.5 with dissociation to hypochlorite ions (OCl^-) occurring above pH 7.5. HOCl is considered to be much more effective as a disinfectant than OCl^-. Chlorine reacts as follows in nitrogen free water: $Cl_2 + H_2O \longrightarrow HOCl + HCl$ (Culp, 1974.; Cramer et al, 1976.; DeMichele, 1976).

In the presence of ammonia, which occurs in many waters, chlorine reacts quite differently. It exists as chloramine, either mono or dichloramine (NH_2Cl, $NHCl_2$). Ammonia is of great concern during disinfection procedures, because municipal wastewater may contain 10 to 30 mg/L of ammonia nitrogen (Culp, 1974). Chlorine disinfection in this situation depends on the pH, temperature, Cl_2 concentration, and ammonia levels. Chlorine, in ammonia laden water, reacts as follows: $NH_4^+ + HOCl \longrightarrow NH_2Cl + H_2O + H^+$

$NH_2Cl + HOCl \longrightarrow NHCl_2 + H_2O$

$NHCl_2 + HOCl \longrightarrow NCl_3 + H_2O$ (Culp, 1974.; Cramer et al, 1976.; DeMichele, 1976).

The HOCl levels must be high in the presence of ammonia for adequate disinfection. Resistant enteric viral inactivation demands a HOCl residual of 1 mg/L maintained for 30 minutes at a pH range of 7 to 7.5 (Culp, 1974). To attain this result, the Cl_2 : NH_3-N ratio must be 10:1; the monochloramine is then oxidized to nitrogen gas (Culp, 1974).

VIRAL PRESENCE, DETECTION, AND REMOVAL FROM WASTEWATER

It has been estimated by Clarke et al (1964) that 20 to 700 viral particles may be present in 100 ml of raw sewage. Some of these infectious agents include poliovirus, Coxsackievirus, echovirus, reovirus, adenovirus, hepatitis virus, and Herpes Simplex virus. There is evidence that human feces contains about one hundred varieties of viruses, of which seventy kinds have been isolated from wastewater in the past two decades (Brown et al, 1974). Associated with this observation, Craun and McCabe (1973) reviewed waterborne disease outbreaks from 1946 to 1970, and concluded that viral, bacterial, and chemically derived illnesses such as infectious hepatitis, gastro-enteritis, typhoid fever, shigellosis, salmonellosis, chemical poisoning, amebiasis, giardiosis, leptospirosis, tularemia, and poliomyelitis, accounted for 72,358 reported cases. It appears that currently used wastewater treatment methods are inadequate for total removal of enteric viruses as well as other microbial health hazards.

There are no standards or regulations for removal of viral pathogens. It is very difficult to detect the low number of viruses present in natural waters, and there is much uncertainty regarding transmission of viruses due to inadequate sampling, enumeration, and identification of the viral agents. Consequently, considerable research is now being performed in an attempt to develop acceptable detection procedures.

Current viral detection and concentration methods were derived from procedures applicable to macromolecules. Certain characteristics of viruses, such as being amphoteric, hydrophilic, exhibiting polarity, having certain sedimentation and adsorption properties allow for physical and chemical isolation and detection techniques. Polyelectrolytes, chemical concentration, ultrafiltration, direct inoculation, ultracentrifugation, freeze concentration, two-phase concentration, hydroextraction, gauze pad filtration, and electrophoresis are a few isolation methods, with the gauze pad procedure now being the most widely used method for detecting viruses (Cookson, 1969.; DeMichele, 1974.; Bier et al, 1967.; Rubenstein et al, 1973.; Shuval et al, 1966.; Sorber et al, 1971.; Wallis et al, 1970.; Buras, 1974.; Zajic, 1971). However, most of the above mentioned procedures are time consuming, expensive, or not practical for large samples of wastewater containing low concentrations of animal viruses. Because of materials needed for animal virus detection, such as animal cell cultures, and trained personnel to maintain viral detection accoutrements, widespread utilization of direct methods for animal viral analysis are presently impractical.

Viral removal can be attained by the utilization of settling tanks, oxidation ponds, activated sludge systems, lime flocculation, carbon contacting stabilization, biological filtration, and diatomaceous earth filtration. The most effective of these is biological filtration through sand (Brown, 1974.; DeMichele, 1974.; Malina et al, 1974.; Nupen et al, 1974.; Stander and VanVuuren, 1969.; Cohen and Kugelman, 1973.; Cookson, 1969). However, the most acceptable and practical means of ensuring safe potable water continues to be disinfection with chlorine.

Disinfection processes have been designed to eliminate bacteria, not viruses. Clarke et al (1964) studying effluents of chlorinated municipal sewage treatment plants found that 39 percent of the samples taken contained detectable virus. Secondary biological treatment, terminated with chlorine disinfection, removes 99 percent of the coliform indicators for bacterial pollution, but it has been demonstrated not to effect complete removal of enteroviruses (Burns, 1967.; Kelley and Sanderson, 1960.; Lothrop and Sproul, 1969). Enteroviruses demand a longer contact time and are much more resistant to chlorine disinfection than coliforms (Weidenkopf, 1958.; Shuval et al, 1966.; Kelley and Sanderson, 1958, 1960a and b.; Cramer et al, 1976). Therefore, the use of a coliform index for determining the quality of wastewater reuse gives no assurance that the chlorine resistant viruses will be inactivated.

Terminal disinfection is a powerful method for cleansing all wastewater of potentially infectious agents. Chlorine disinfection is the most feasible and effective microbicide used today. Even though chlorine has been used for 50 years, its application by wastewater facilities could be further maximized.

A possible substitute for chlorine is ozone. Ozone is a more effective microbicide and is not affected by pH or ammonia. It also has beneficial affects upon wastewater's color, taste, and odor (Culp, 1974).

CYANOPHAGE AS INDICATORS OF WATER QUALITY

The objective of this study is to further substantiate a previous proposal to use cyanophage as possible indicators of animal viruses and coliforms in wastewater (Smedberg and Cannon, 1976). Determination of cyanophage chlorine resistance and serological typing of cyanophage LPP-1 and LPP-2, isolated from wastewater, were performed in hopes of obtaining additional data to support the use of cyanophages as an indicator of water quality. New indicator methods need to be proven as reliable. These methods should be sensitive enough to detect small quantities of virus, inexpensive, and simple enough to be used in routine monitoring of water. The proposed model is based on the blue-green algal viruses LPP-1 and LPP-2, that have been isolated from waters world wide, and are virulent for the blue-green alga genera Lyngbya, Plectonema, and Phormidium (Safferman and Morris, 1963.; Safferman et al, 1969.; Safferman and Morris, 1967).

Cyanophage detection procedures can be performed easily, inexpensively, and with a high degree of accuracy in the detection of small quantities of virus. They can be isolated year round from wastewater and are more resistant to chlorination than are animal viruses. The use of cyanophages as indicators of water quality can be a more efficient model for indirectly determining the reliability of disinfection processes.

MATERIALS AND METHODS

WASTEWATER TREATMENT PROCEDURES AT NORTH BUFFALO CREEK SEWAGE PLANT

Wastewater samples were taken biweekly from August 28, 1975 through May 19, 1976 at the North Buffalo Creek Sewage Treatment Plant, Greensboro, North Carolina. The plant employs primary and secondary treatment with terminal chlorine disinfection. Domestic wastewater makes up the bulk of water treated with a small percentage of industrial discharge processed.

The wastewater is screened initially through a grit chamber to remove larger debris and is allowed to remain two hours in settling tanks. It is then passed through two separate trickling filters and channeled to activated sludge basins where continuous aeration is maintained. Aeration continues for eight hours followed by another settling period in clarification tanks for two hours. This clarification process is followed by terminal disinfection where an attempt is made to maintain a residual chlorine level of 0.5 mg/L for 20 minutes as required by EPA standards.

Four composite samples were taken every two weeks from the inflow, following primary settling tanks, following trickling filtration, and after chlorination. Collections were transported immediately to the laboratory and cyanophage detection procedures were initiated.

PLECTONEMA BORYANUM CULTIVATION

The LPP cyanophage host range includes members of the genera Lyngbya, Plectonema, and Phormidium. Plectonema boryanum was chosen for the cyanophage host, because of its more desirable culture characteristics. Cyanophage detection assays were performed on the blue-green alga P. boryanum strain 594 of the Indiana University culture collection. Stock cultures were grown and maintained in Modified Chu #10 medium, at 25°C with a "cool white" fluorescent light source (100 - 120 ft-c) illuminating the culture vessels. Plectonema is a filamentous alga that grows confluently throughout the medium to an approximate density of 10^7 cells/ml (Smedberg and Cannon, 1976). The culture medium stock solutions are composed of 23.3 gm/L $Ca(NO_3)_2 \cdot 4H_2O$; 1 gm/L K_2HPO_4; 2.5 gm/L $MgSO_4 \cdot 7H_2O$; 2.0 gm/L Na_2CO_3; 16.6 gm/L $NaNO_3$; 5.9 gm/L $Na_2SiO_3 \cdot 9H_2O$; 0.35 gm/L ferric citrate; 0.35 gm/L citric acid; and trace elements. The trace elements stock solution is composed of 0.001 gm/L $CuCl_2 \cdot 2H_2O$; 0.04 gm/L $ZnCl_2$; 0.24 gm/L H_3BO_4; 0.002 gm/L $CoCl_2 \cdot 6H_2O$; and 0.14 gm/L $MnCl_2 \cdot 4H_2O$. Preparation of 1 liter of medium consists of 2.5 ml $Ca(NO_2)_2 \cdot 4H_2O$; 10 ml K_2HPO_4; 10 ml $MgSO_4 \cdot 7H_2O$; 10 ml $NaCO_3$; 17.5 ml $NaNO_3$; 10 ml $Na_2SiO_3 \cdot 9H_2O$; 10 ml of ferric citrate; 10 ml citric acid; and 10 ml of trace elements added to 1000 ml of distilled water. The thirteen 125 ml flasks, containing 50 ml of sterile Modified Chu #10 medium used for each sampling, were aseptically inoculated with 5 ml of host algae and allowed to grow for 7 days under the fluorescent lights before being used for cyanophage detection.

MAINTENANCE AND PREPARATION OF CYANOPHAGE STOCK

Two hundred milliliters of medium dispensed into a one liter Erlenmyer flask was inoculated with 10 ml of Plectonema boryanum. Culture vessels were incubated for 2 to 3 weeks at 25°C under continuous illumination. After the appropriate incubation period, LPP-1 and LPP-2 at titers of 10^8 plaque forming units per milliliter (PFU/ml), obtained from R. S. Safferman, National Environmental Research Center, Cincinnati, Ohio, were added separately to the two flasks. Three to seven days of incubation were required for complete lysis of the host. Lysates were vacuum filtered through 0.45 micron membrane filters, (Millipore Corporation) and stored at 4°C. The cyanophage titers varied between 10^7 - 10^8 PFU/ml.

ASSAYING PROCEDURES

Assays for cyanophages have been developed from modifications of Adams' (1950) bacteriophage methods (Safferman and Morris, 1964). Petri plates were prepared for assaying purposes by layering the bottom with 10 ml of 1.5% Modified Chu agar. The over-lay medium consists of 2.5 ml of 1% melted Chu agar, 2 ml of a 3 week old culture of P. boryanum, and 0.5 ml of appropriately diluted cyanophage. All virus samples were diluted at tenfold increments in a sterile salt solution containing 0.42 g of $MgCl_2 \cdot 6H_2O$; 5.85 g of NaCl per 1000 ml of distilled water. Plates were incubated at 25°C, under fluorescent lights (100 - 120 ft-c) for 2 to 3 days before plaques appeared. P. boryanum grows evenly over the prepared plates making any lysis easily observed. Plaques appear clear with variation in diameter size from 0.1 mm to 8 mm. Reliability

of cyanophage plating procedures are comparable with the efficiency reported for bacterial viruses (Safferman and Morris, 1964).

CYANOPHAGE DETECTION PROCEDURE

Thirteen 125 ml Erlenmyer flasks, containing 50 ml of Plectonema boryanum, were prepared seven days prior to sampling dates, with a resulting cell density of approximately 10^7 cells/ml. Fifty milliliters of wastewater from each sampling station were inoculated into each of three flasks for each sampling point. One flask was retained as a control. This is analogous to the multiple tube fermentation test for coliforms as described in Standard Methods (1965.; Smedberg and Cannon, 1976).

Flasks were incubated for three to four days. If lysis of the host occurred it was considered a positive presumptive test for cyanophages. To verify the presumptive test, 10 ml aliquots were taken from each lysed flask, one ml of chloroform was added and the total shaken for one minute. Chloroform was employed to remove any agents, such as protozoans or bacteria, that may also lyse algae. After allowing the chloroformed lysate to settle for thirty minutes, one milliliter was transferred into Micro-Fernbach flask containing 15 ml of P. boryanum. If lysing occurs after an incubation period of one to three days, this is considered a positive confirmed test for cyanophage.

Another 10 ml aliquot was taken from the Micro-Fernbach lysate and chloroformed. Cyanophage titers were then determined by the soft agar method. Duplicate plates were made of each sample with plagues forming after an incubation period of 48 - 72 hours. Only those plates with 30 to 300 plaques were used to determine PFU/ml.

CYANOPHAGE ANTISERA PREPARATION

To prepare antisera against LPP-1 and LPP-2 cyanophage, rabbits were used. One milliliter samples of LPP-1 (2.30×10^8 PFU/ml) and LPP-2 (2.02×10^7 PFU/ml) were emulsified in two milliliters of Freund's Complete Adjuvant (Difco). All injections were administered intraperitoneally with 3 ml of the adjuvant-virus mixture injected. Injections were made at two week intervals, with cardiac punctures performed two weeks after the second injection. Fifty milliliters of blood were extracted and centrifuged for thirty minutes at 10,000 x g, the serum was then removed and stored at 4°C (Carpenter, 1975).

SEROLOGICAL TYPING PROCEDURE

Cyanophage LPP-1 and LPP-2 are not easily discernible from each other by general morphology (Safferman et al, 1969.; Sherman, 1970 a and b). They both possess hexagonal capsids containing DNA as the nucleic material, and a short tail, about one-fourth the diameter of its head (Schneider et al, 1964.; Smith et al, 1966.; Luftig and Haselkorn, 1967, 1968). However, cyanophage LPP-1 is serologically distinguishable from LPP-2 (Safferman et al, 1969.; Safferman and Morris, 1967).

A total of 672 pure plaques were picked for subsequent antigenic study. The total was made up of 48 plaques from each positive sampling date. This represents a confidence level of 92% (Appendix A). Each isolated plaque picked was aseptically transferred to a 25 ml Micro-Fernbach flask containing 15 ml of P. boryanum, cell density of 10^7/ml. When lysis occurred after incubation, 10 ml aliquots were chloroformed and allowed to settle for 30 minutes. From the lysates, cyanophage

assays were performed to determine if viable particles were present.

For each plaque to be tested, two sterile test tubes were used; one containing 0.5 ml of the LPP-1 antiserum and the other containing 0.5 ml of the LPP-2 antiserum. The antisera was diluted 1:10 in sterile 0.9% saline before use. Each of the two tubes received 0.5 ml of the virus suspension, diluted as previously described, and were incubated for thirty minutes. After the reaction period, cyanophage assays were performed. In cases where neutralization of virus particles did not occur plaque formation appeared.

In addition to the testing of each isolated plaque, neutralization tests were performed on concentrated stock samples of LPP-1 (2.30×10^8 PFU/ml) and LPP-2 (2.02×10^7 PFU/ml) to measure the effectiveness of the antisera. One-half milliliter of undiluted cyanophage was mixed with 0.5 ml of homologous antiserum and allowed to react for 30 minutes. One-half milliliter samples were taken from the LPP-1 and LPP-2 flask and separately assayed, as previously described, every five minutes for a total of thirty minutes.

WASTEWATER TREATMENT PRIOR TO CHLORINE INACTIVATION TEST

Biologically treated wastewater was obtained from the North Buffalo Creek Wastewater Treatment Plant in Greensboro, North Carolina. Samples were taken from clarification tanks following the activated sludge treatment. The wastewater was autoclaved for thirty minutes at 121°C and 15 psi, then vacuum filtered through Whatman #1 filter paper. The pH was adjusted between 7 to 7.5 with 1 M NaOH or 1 M HCl. For consistency and experimental reliability, the entire sample of wastewater needed was collected the same day, treated, and stored at

room temperature for subsequent chlorination test. This wastewater is not identical to raw wastewater, but chemically very similar (Cramer et al, 1976). Actual wastewater was preferred over glass distilled water for the studies because results can be considered more relevant to actual disinfection processes occurring in waste treatment plants.

RESIDUAL CHLORINE DETERMINATION

Residual chlorine was measured by the orthotolidine method. This method is used to measure both free and combined available chlorine. The principle of this method is to obtain the correct color development between chlorine and orthotolidine. If the wastewater does not contain more than 0.3 mg/L iron, 0.01 mg/L manganic manganese, and 0.1 mg/L nitrate nitrogen the development of the yellow color may be assumed to be attributable to chlorine (Standard Methods, 1965).

One hundred milliliter portions of autoclaved, pH adjusted, filtered wastewater were dispensed into 125 ml Erlenmyer flask. One milliliter of orthotolidine was added plus liquid chlorine. Transmittance measurements at 460 nm were taken spectrophotometrically (Spectronic 20 Bausch & Lomb Co.), five minutes after the addition of chlorine, with maximum color development usually occurring within the first five minutes (Standard Methods, 1965.; Brown, 1976). Residual chlorine was determined by comparing percent transmittance readings to a semi log graph experimentally derived using known chlorine standards (Appendix B.; Brown, 1976). The inactivation experiments were performed at a residual chlorine level of 2.5 mg/L.

The orthotolidine solution was prepared by dissolving 1.35 grams of orthotolidine dihydrochloride in 500 ml of distilled water. This solution was added to a mixture of 150 ml of concentrated HCL and 350 ml of distilled water. It was stored in amber bottles at room temperature (Standard Methods, 1965). The liquid chlorine was obtained from the North Buffalo Creek Wastewater Treatment Plant, stored in an amber bottle at 4°C.

CHLORINE INACTIVATION PROCEDURES

The chlorine inactivation procedure which was chosen models chlorine inactivation procedures for studies of the bacteriophage f_2 and poliovirus III (Cramer et al, 1976). All experiments were performed at room temperature, and the residual chlorine levels were monitored by the orthotolidine method, as previously described, after a prereaction period of 30 minutes.

Ninety seven milliliters of the pretreated wastewater were added to a 250 ml Erlenmyer flask. To this was added 20 mg/L of liquid chlorine and allowed to react for 30 minutes. After the prereaction period, one milliliter of cyanophage was added. The serological type and virus titer were known. Samples were taken from the flask at five minute intervals up to twenty five minutes of disinfection time, and cyanophage assays were performed. After forty-eight to seventy-two hours, plaques appeared and percent survival was determined by a comparison of the initial titer to recovery titer.

Chlorine resistance was also determined for stock LPP-1 and LPP-2. This was done to determine if any significant difference exists in the degree of resistance to chlorine disinfection of stock and wastewater plant isolated cyanophages. Plus, pure strains of cyanophages were inoculated into pretreated wastewater with no chlorine and allowed a twenty five minute exposure time. Assays were then performed to determine if cyanophages were inactivated in unchlorinated wastewater.

After the chlorine inactivation experiments were finalized and tabulated, composite averages of the percent inactivations were used to determine Ct.99 figures (Appendix C). Ct.99 represents the time and dosage needed for 99 percent inactivation; Chlorine dose mg/L x time min. = Ct.99 (Cramer et al, 1976).

RESULTS

CYANOPHAGE PRESENCE AT SAMPLING POINTS

Cyanophages were isolated throughout the wastewater treatment plant, with a greater number isolated during warmer months and fewer during colder months, plus a deviation from the expected occurring during early spring. Results from the cyanophage presumptive and confirmed tests, as well as cyanophage titers are recorded in table 4. The bar graph (Figure 1) illustrates the cyanophage distribution throughout the ten months of sampling.

Positive presumptive and confirmed test results were usually obtained within seventy-two hours. Lysing of the host occurred more often in the first three stations than in the fourth, but a significant number of positive confirmed tests did occur after chlorine disinfection. The predominant station for isolation of cyanophage was station three, which follows the trickling filter. This phenomenon is probably attributable to the blue-green algae present on the rocks, which maintain a higher titer of cyanophage than in other points of the treatment plant.

Several negative confirmed tests were noted in early spring after positive presumptive tests. This may result from chemical toxins, protozoans, or an ecological case of destructive parasitism (Safferman, 1973). The March 11 sampling also demonstrated a deviation from previous results. Lysis occurred in the flasks inoculated with chlorinated effluent, but no positive results were observed in any of

the first three stations. Apparently cyanophage survived the disinfection process, but due to inherent sampling error, no manifestation of cyanophages were noted before the fourth station. During winter months there was a significant decline in the number of cyanophage detected. This is to be expected since the cyanophage host, blue-green algae, are not as prevalent during colder months.

Plectonema boryanum and the LPP cyanophage are not easily affected by pH variations. Both have a tolerance range between 5 to 11 (Safferman and Morris, 1964). The pH readings for the wastewater samples were within the tolerance range, varying between 7 to 7.5.

CYANOPHAGE SEROLOGICAL TYPING RESULTS

From the strains of cyanophage obtained from wastewater samples, serological type LPP-2 predominated over LPP-1 about 90% to 10% respectively. On three occasions, the yields of LPP-2 reached a high of 100% and twice a low of 75%. Because a great deal of negative confirmed tests were recorded during spring months, no evaluation can be given for this period. Results from each sampling date are presented in table 5. The antisera were tested for effectiveness by using known titers of stock virus LPP-1 and LPP-2. It was found that the viral particles were completely neutralized within the first five minutes.

TABLE 4

RESULTS FROM CYANOPHAGE DETECTION PROCEDURE

Date	Sample Point	Number of Lysed Flasks (Presumptive Test)	Number of Lysed Flasks (Confirmed Test)	Cyanophage PFU/ml (Confirmed)
Aug. 28 1975	1 Inflow	3	3	7.50×10^5
	2 Before Trickling Filter	3	3	2.12×10^6
	3 After Trickling Filter	3	3	1.02×10^6
	4 Effluent	3	3	1.08×10^6
Sept. 10	1	3	3	5.80×10^6
	2	3	3	1.72×10^6
	3	3	3	2.90×10^6
	4	0		
Sept. 24	1	3	3	4.40×10^6
	2	3	3	3.00×10^7
	3	3	3	4.00×10^6
	4	3	3	1.70×10^7
Oct. 8	1	1	1	2.80×10^7
	2	3	3	1.64×10^7
	3	2	2	3.00×10^7
	4	0		
Oct. 22	1	3	3	1.20×10^6
	2	3	3	4.60×10^5
	3	3	3	1.80×10^6
	4	2	2	1.40×10^6

TABLE 4

(Continued)

Date	Sample Point	Number of Lysed Flasks (Presumptive Test)	Number of Lysed Flasks (Confirmed Test)	Cyanophage PFU/ml (Confirmed)
Nov. 5	1	3	3	1.20×10^8
	2	3	3	3.30×10^8
	3	3	3	6.80×10^8
	4	0		
Nov. 19	1	1	1	9.80×10^7
	2	2	2	4.80×10^6
	3	1	1	7.80×10^6
	4	1	1	8.00×10^6
Dec. 4	1	0		
	2	3	3	6.40×10^6
	3	3	3	7.20×10^6
	4	0		
Dec. 18	1	1	1	1.83×10^5
	2	3	3	9.60×10^5
	3	3	3	9.80×10^5
	4	0		
Dec. 31	1	0		
	2	2	2	3.18×10^6
	3	3	3	1.44×10^6
	4	0		

TABLE 4

(Continued)

Date	Sample Point	Number of Lysed Flasks (Presumptive Test)	Number of Lysed Flasks (Confirmed Test)	Cyanophage PFU/ml (Confirmed)
Jan. 15 1976	1	0		
	2	2	2	6.40×10^5
	3	1	1	6.20×10^5
	4	1	1	7.40×10^5
Jan. 28	1	0		
	2	0		
	3	1	1	6.20×10^6
	4	0		
Feb. 11	1	0		
	2	0		
	3	2	2	7.40×10^6
	4	0		
Feb. 25	1	3	0	
	2	0		
	3	0		
	4	0		
Mar. 11	1	0		
	2	0		
	3	0		
	4	3	3	4.80×10^6

TABLE 4

(Continued)

Date	Sample Point	Number of Lysed Flasks (Presumptive Test)	Number of Lysed Flasks (Confirmed Test)	Cyanophage PFU/ml (Confirmed)
Mar. 25	1	1	0	
	2	0		
	3	0		
	4	0		
Apr. 7	1	3	0	
	2	0		
	3	0		
	4	0		
Apr. 21	1	3	0	
	2	0		
	3	0		
	4	0		
May 5	1	1	0	
	2	1	0	
	3	3	0	
	4	0		
May 19	1	3	0	
	2	3	0	
	3	3	0	
	4	0		

figure 1

CYANOPHAGE DISTRIBUTION

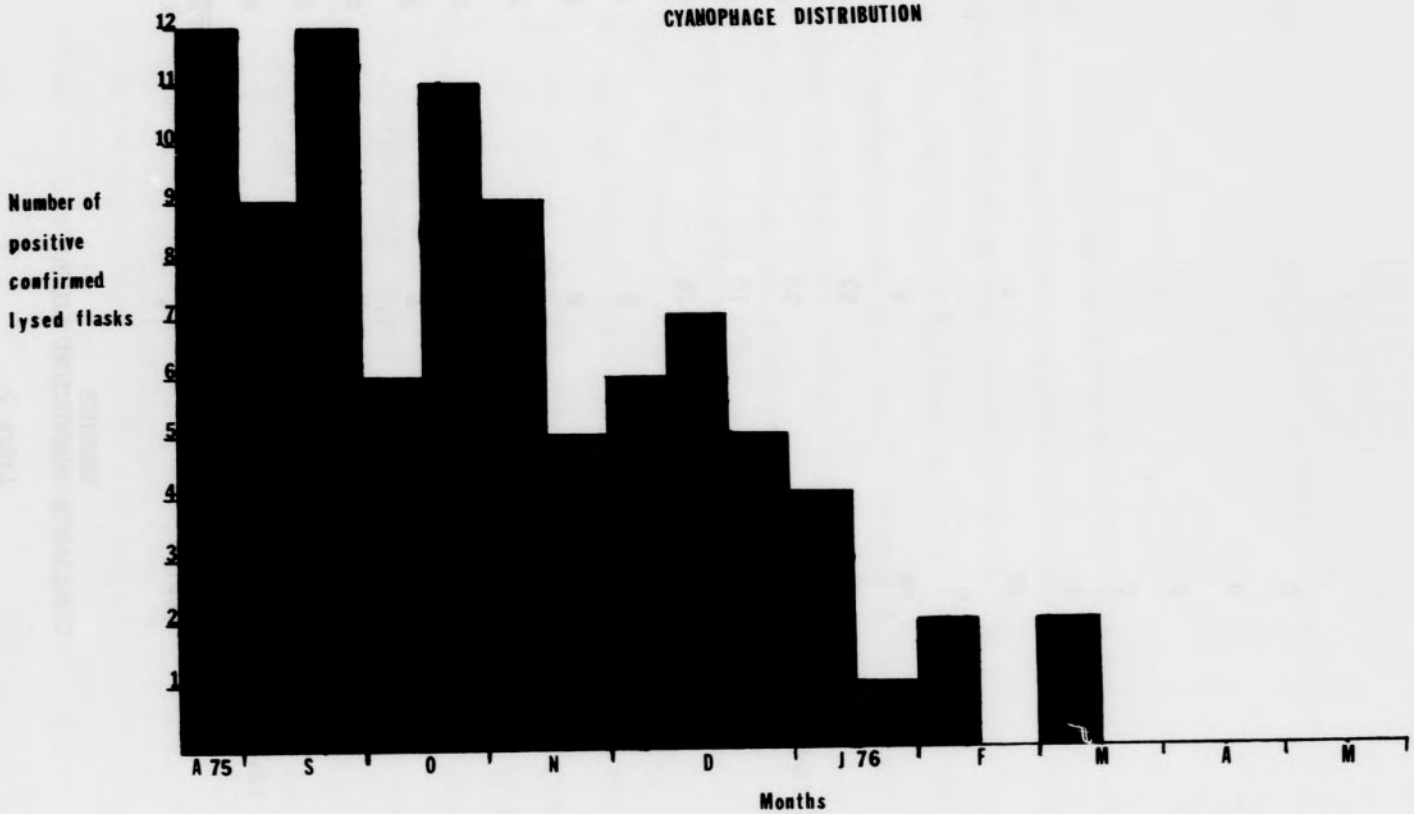


TABLE 5

CYANOPHAGE SEROLOGICAL TYPING RESULTS

Date	Sample Size	% LPP-1	% LPP-2
Aug. 28, 1975	48	8	92
Sept.10	48	25	75
Sept.24	48	2	98
Oct. 8	48	2	98
Oct. 22	48	17	83
Nov. 5	48	0	100
Nov. 19	48	0	100
Dec. 4	48	8	92
Dec. 18	48	10	90
Dec. 31	48	10	90
Jan. 15, 1976	48	13	87
Jan. 28	48	25	75
Feb. 11	48	8	92
Feb. 25	0		
Mar. 11	48	0	100
Mar. 25	0		
Apr. 7	0		
Apr. 21	0		
May 5	0		
May 19	0		

CHLORINE INACTIVATION

Stock LPP-1 (2.25×10^8 PFU/ml) and LPP-2 (1.16×10^7 PFU/ml) were significantly affected by a combined chlorine residual of 2.5 mg/L. Ninety-nine percent kill for both LPP-1 and LPP-2 was attained after 15 to 20 minutes of exposure (Appendix C). No significant loss of titer was noted when stock cyanophages were incubated in unchlorinated wastewater. One hundred percent survival was observed after a 25 minute contact period. Ninety-nine percent cyanophage inactivation at 15 minutes of exposure time represents a Ct.99 of 300.

Cyanophages isolated from wastewater showed a much higher resistance to combined chlorine. Ninety-nine percent kill was not attained during the 25 minute contact period (Appendix C). The Ct.99 figures for wastewater isolates will be substantially higher than those of stock cyanophage. A comparison between the serologically distinct LPP-1 and LPP-2 in their resistance to chlorine disinfection shows no noteworthy difference. Stock or wastewater isolated strains of both LPP-1 and LPP-2 were inactivated at similar rates (Figure 2,3). The difference of resistance to combined chlorine disinfection between identical strains of stock cyanophages and cyanophages isolated from wastewater treatment plants may be seen in figure 2 and 3.

figure 2

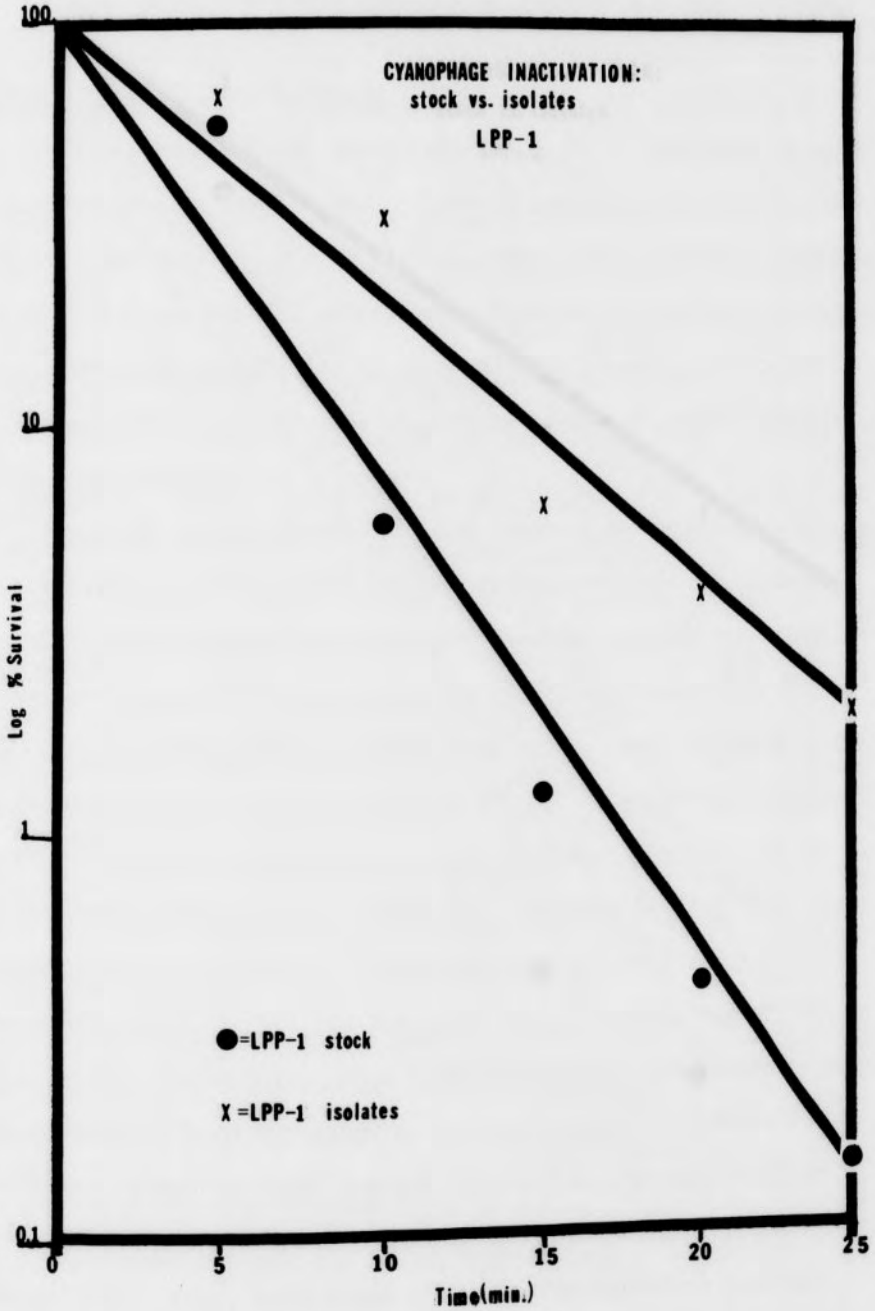
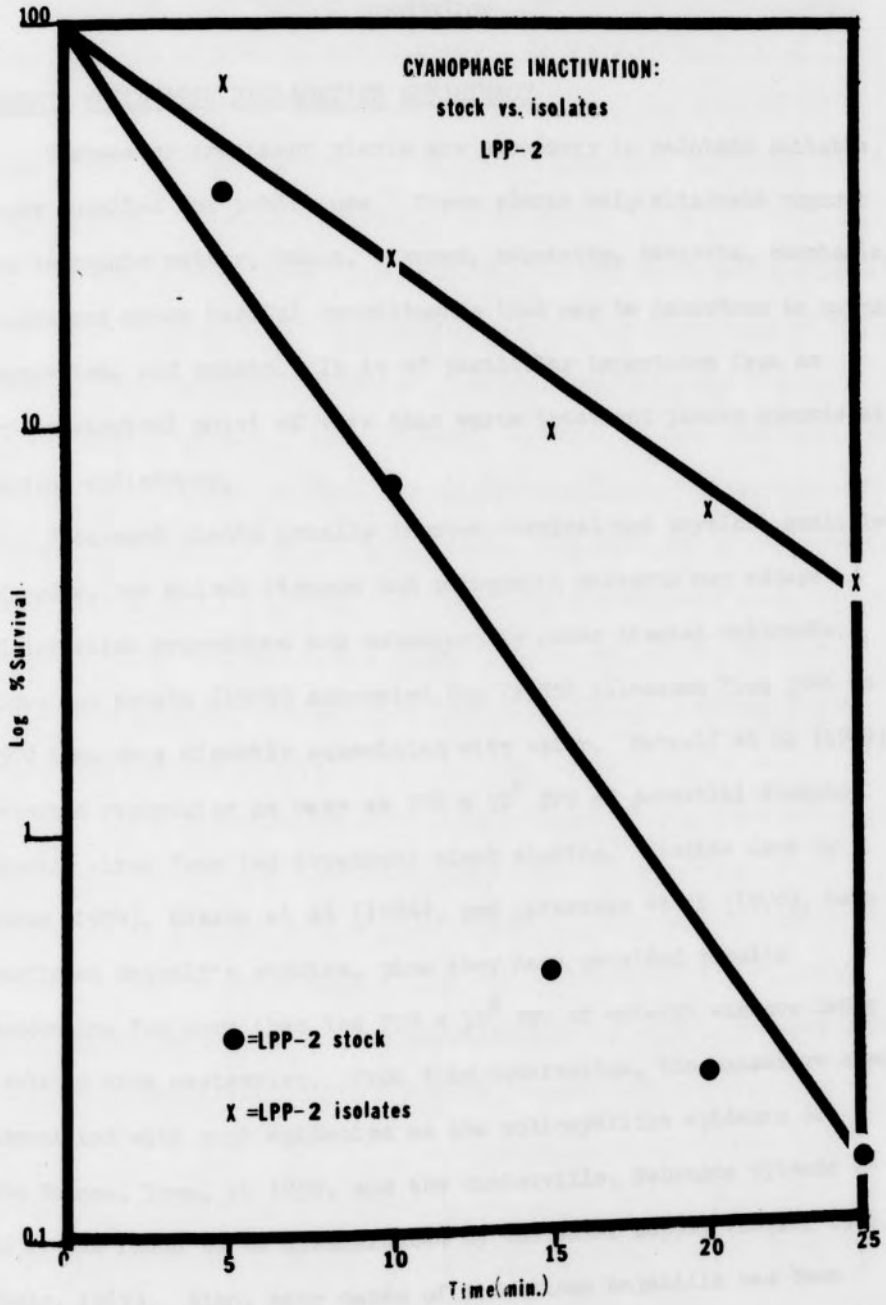

CYANOPHAGE INACTIVATION:
stock vs. isolates
LPP-1
100
10
1
0.1
Log % Survival
0
5
10
15
20
25
Time(min.)
●=LPP-1 stock
X =LPP-1 isolates

figure 3


CYANOPHAGE INACTIVATION:
stock vs. isolates
LPP-2
100
10
1
0.1
Log % Survival
0
5
10
15
20
25
Time(min.)
●=LPP-2 stock
X =LPP-2 isolates

DISCUSSION

PRESENT WASTEWATER RECLAMATION EFFICIENCY

Wastewater treatment plants are necessary to maintain suitable water supplies for public use. These plants help eliminate organic and inorganic matter, odors, viruses, helminths, bacteria, chemicals, toxins and other harmful constituents that may be hazardous to animals, vegetation, and humans. It is of particular importance from an epidemiological point of view that waste treatment plants operate at maximum efficiency.

Treatment plants usually improve chemical and physical qualities of water, but animal viruses and pathogenic bacteria may escape disinfection procedures and subsequently cause disease outbreaks. Crown and McCabe (1974) accounted for 72,358 illnesses from 1946 to 1970 that were directly associated with water. Metcalf et al (1974) reported recovering as many as 778×10^6 PFU of potential disease-causing virus from two treatment plant studies. Studies done by Buras (1974), Clarke et al (1964), and Grinstein et al (1970), have confirmed Metcalf's studies, plus they have provided results accounting for more than the 778×10^6 PFU of enteric viruses being isolated from wastewater. From this observation, the causative agent associated with such epidemics as the poliomyelitis epidemic of Des Moines, Iowa, in 1959, and the Huskerville, Nebraska episode in 1957, was found to be disseminated by the water supply (Zajic, 1971,; Mosly, 1967). Also, many cases of infectious hepatitis has been

attributed to contaminated water within the past seventy years (Mosly, 1967). In addition, mortality rates in unindustrialized countries are as much as one hundred times greater than the more economically advanced countries. There seems to be a direct correlation between the quality of water management and mortality rates due to contaminated water (Miller, 1962).

As can be seen from table 6 taken from the North Buffalo Creek Wastewater Treatment Plant's monthly report, the percentage of BOD is lowered and suspended solids are removed significantly. The pH range remains between 7 and 7.5 where chlorine is most effective, but the nitrogen content exceeds the acceptable limits for maximum microbicidal effectiveness. Associated with this, as can be seen in table 6, the fecal coliform indicators are consistantly found in the effluent after chlorination. There are also occasions, notably September, October, and November when the chlorine residual drops below the acceptable limits.

The above observations seem to be more the norm rather than an exception. Wastewater treatment plants seem to operate only marginally in regards to the real hazards to man, such as bacterial, parasitic, and viral threats. Even though bacterial and parasitic hazards can be removed by present wastewater treatment, viruses require more extensive treatment for complete inactivation.

A second defense exists to protect the public against hazardous enteric viruses. When outside the body the viruses are not in an acceptable environment and may be inactivated by environmental stresses. Even though this extra safety element exists, as the population escalates the number of potential disease causing viruses reaching water supplies will definitely become too great to be destroyed by antiquated reclamation facilities.

SIGNIFICANCE OF CYANOPHAGE PRESENCE

It would be reasonable to assume that cyanophage presence is directly associated with its host population. In this study cyanophage presence in wastewater was high during summer and early fall months but after the decline of cyanophages in winter months no substantial increase of the viruses occurred during the warmer spring months. Several negative confirmed tests for cyanophage were recorded up through May. This observation corresponds with observations made by Safferman (1973) in his study of cyanophage distribution throughout the year in an Arkansas waste stabilization pond. He expected a high titer of cyanophage in July, August, and September when the algal host would be flourishing, but he obtained very low titers of the virus. No concrete evidence exists, as yet, to explain this phenomenon. One theory deals with the pathological effects of the virus. The hosts' dominance during different times of the year could be temporarily depleted by the virus resulting in a substantial evanescent decline in the virulent cyanophages (Safferman and Morris, 1967,1973). This occurrence ultimately gives way to a balanced state between alga and cyanophage.

TABLE 6

NORTH BUFFALO CREEK WASTEWATER TREATMENT EFFICIENCY

Date	5 day 20°C % removal BOD	Overall suspended solids % removal	Kjeldahl nitrogen effluent mg/L	Average composite pH	Residual chlorine	Effluent average/100 ml fecal coliform
Aug. 1975	94.6	90.9	NA	7.3	0.5	NA
Sept.	92.5	NA	12.03	7.2	0.23	4340
Oct.	84.9	76.5	12.16	7.3	0.17	14674
Nov.	91.8	79.7	8.67	7.3	0.28	1345
Dec.	94.4	98	13.59	NA	0.96	0
Jan. 1976	91.3	78.2	12.88	7.3	1.27	5666
Feb.	94.9	81.8	14.68	NA	1.6	18
Mar.	95.7	86	16.88	7.2	1.8	25
Apr.	93.6	83.6	17.53	7.3	1.04	476
May	NA	NA	13.78	NA	NA	3375

*NA= Not available

On all the dates that negative confirmed tests for cyanophages were recorded, positive presumptive tests were noted. This is probably due to protozoans or chemical toxins. If so, the cyanophages may also be inactivated by unknown constituents which account for the negative confirmed tests.

It is known that the two cyanophages LPP-1 and LPP-2 are distinguishable serologically, but not morphologically. Of the cyanophages isolated from wastewater samples throughout the ten months, serological results indicated LPP-2 predominated over LPP-1 about nine to one. LPP-1 was the first of the two cyanophages to be isolated from wastewater stabilization ponds by Safferman (1973,1963), but it seems more likely that LPP-2 would have been isolated initially if they do predominate so markedly over LPP-1. However, there could be a geographical difference in the distribution of the two cyanophages. Because of the negative confirmed tests for cyanophage during the spring months, and no cyanophage to more thoroughly study serologically, it would be premature to suppose that complete dominance by either LPP-1 or LPP-2 occurs. Serological determinations of cyanophages need to be continued for a longer period of time; virus samples need to be taken over a wider geographical range. This will provide statistical evidence to compensate for seasonal and ecological fluctuation of cyanophages throughout the year.

The occurrence of a greater number of cyanophage during warmer months parallels an observation of Fenner et al (1974) that animal viruses are also more dominant during warmer periods of the year. Cyanophage detection procedures may be utilized to indicate possible

animal virus presence or to determine removal efficiencies of treatment processes (Smedberg and Cannon, 1976.; Shane et al, 1967). The multiple flasks and multiple tube methods are comparable in reliability as determined by MPN indexes (Smedberg and Cannon, 1976). As the multiple tube method is an indirect indicator of the possible presence of bacterial pathogens, so too is the multiple flasks method an indirect method to determine possible animal virus presence.

The detection materials for animal viruses are expensive and difficult to maintain and there is a risk associated with the manipulation of virulent animal viruses. Present isolation techniques are impractical, because some of the methods are unreliable and expensive. Also, there are complications associated with the size of the water samples to be processed. In contrast, cyanophage detection materials and procedures are inexpensive, reliable, and easy to maintain in wastewater treatment laboratories; and safe because the cyanophage is not pathogenic to man. Therefore, cyanophages seem to be more reliable indicators of water quality than those presently used.

SIGNIFICANCE OF CYANOPHAGES' CHLORINE RESISTANCE

In this study, inactivation of cyanophages can be considered to be attributable to combined chlorine species because of the prereaction period. As reported by Kelley and Sanderson (1958), animal viruses show a chlorine resistance according to the following order; poliovirus 1(MK 500) and poliovirus 3 > Coxsackie B1 > poliovirus 2 > poliovirus 1 > Coxsackie B5. Cyanophages are more resistant to combined chlorine than the most resistant animal virus. Also, results presented can be compared to other studies performed similarly (Table 7).

TABLE 7

Comparative Resistance of Various Organisms to Combined Chlorine (Cramer et al,1976)

Organism	Chlorine dose (mg/L)	x	Time (min.)	=	Ct.99
f_2	30		68		2,040
Simian cysts	13.7		92		1,260
Poliovirus III	30		9.25		278
Poliovirus I	27		9.5		256
Coliforms	4		11		44

Stock cyanophages were inactivated by a chlorine dose of 20 mg/L in 15 minutes, this represents a Ct.99 figure of 300. Even though this is higher than poliovirus III with a Ct.99 of 278 it still is not as high as Ct.99 figures for cyanophages isolated from wastewater. Ninety-nine percent inactivation was not attained during the 25 minute contact time, so actual Ct.99 figures may be as high as 600. Cyanophage isolated from the environment seem more resistant to chlorine disinfection than stock strains of cyanophage and animal viruses.

The use of cyanophages as an indicator of enteric virus removal from wastewater does not require that the cyanophages have to be more resistant to chlorination than the enteric viruses, but should show a similarity. Cyanophages appear to be more resistant which makes them likely candidates as a virus indicator. Cramer et al (1976) suggested the use of f_2 bacteriophage as a virus indicator. F_2 is much more resistant to chlorine disinfection than cyanophages, but f_2 has no specific host (Cramer et al, 1976). The host Escherichia coli may

have many T-phages that attack it. Because of this, the wastewater treatment facilities would have to seed influents with titers of 10^6 PFU/ml to compensate for plaques caused by background virus. However, cyanophages are specific for blue-green algae and no other virus is known to attack the blue-green algae used in this study.

CONCLUSION

Cyanophages are found year round in wastewater, they are more resistant to chlorine disinfection than animal viruses, and their assays are reliable, inexpensive and easy to perform. All the present empirical information associated with the utilization of cyanophages as pollution indicators; bacterial and viral, seem to substantiate the use of cyanophages as a more reliable parameter to determine the effectiveness of wastewater disinfection processes. If cyanophages are removed from wastewater by disinfection processes it can be assumed that pathogenic bacteria and viruses are also inactivated.

BIBLIOGRAPHY

Bier, M., Bruchner, G. C., Cooper, F. C. and Ray, H. E. 1967. Concentration of Bacteriophage by Electrophoresis. p. 57-74. In Gerald Berg (ed.) Transmission of Viruses by the Water Route. Interscience Publishers, New York.

Brown, K. 1976. Personal Communication. North Buffalo Creek Wastewater Treatment Plant, Greensboro, N. C.

Brown, T. S., Malina, J. F., Moore, B. D., Sagik, B. D. 1974. Virus Removal by Diatomaceous Earth Filtration. p. 129. In J. F. Malina and B. P. Sagik (ed.) Virus Survival in Water and Wastewater Systems. Center for Research in Water Resources, University of Texas, Austin, Texas.

Buras, N. 1974. Recovery of Viruses from Wastewater and Effluent by the Direct Inoculation Method. Water Research. 8:19-22.

Burns, R. W. and Sproul, O. J. 1967. Virucidal Effects of Chlorine in Wastewater. J. Water Poll. Control Fed. 39:1834-1849.

Carpenter, P. L. 1975. Immunology and Serology. p. 312-314. W. B. Saunders Company, Philadelphia-London-Toronto.

Clarke, N. A., Berg, G., Kabler, P. W. and Chang, S. L. 1964. Human Enteric Viruses in Water. Source, Survival, and Removability. p. 523-536. In W. W. Eckenfelder (ed.) Advances in Water Pollution Research. Vol. 2., MacMillan Co. New York.

Cohen, J. M. and Kugelman, I. J. 1973. Wastewater Treatment-Physical and Chemical Methods: Annual Literature Review. J. Water Poll. Control Fed. 45:1027-1038.

Cookson, J. T. 1969. Mechanism of Virus Adsorption on Activated Carbon. Jour. Amer. Water Works Assn. 61:52-56.

Cramer, W. N., Kawata, K. and Kruse, C. W. 1976. Chlorination and Iodination of Poliovirus and f_2. J. Water Poll. Control Fed. 48:61-76.

Craun, G. F. and McCabe, L. J. 1973. Review of the Causes of Waterborne-Disease Outbreaks. Jour. Amer. Water Works Assn. 65:74-84.

Culp, R. L. 1974. Breakpoint Chlorination for Virus Inactivation. p. 158-164. In J. F. Malina and B. P. Sagik (ed.) Virus Survival in Water and Wastewater Systems. Center for Research in Water Resources, University of Texas, Austin, Texas.

DeMichele, E. 1974. Water Reuse, Virus Removal and Public Health. p. 47, 54. In J. F. Malina and B. P. Sagik (ed.) Virus Survival in Water and Wastewater Systems. Center for Research in Water Resources, University of Texas, Austin, Texas.

DeMichele, E. 1976. Personal Communications.

Fenner, F., McAuslan, B. R., Mims, C. A., Sambrook, J., White, D. O. 1974. In The Biology of Animal Viruses. Academic Press, Inc., New York.

Grinstein, S., Melnick, J. L. and Wallis, C. 1970. Virus Isolation From Sewage and From a Stream Receiving Effluents of Sewage Treatment Plants. Bulletin World Health Organization. 42:291-296.

Holder, D. 1976. Personal Communications. Superintendent of North Buffalo Creek Sewage Treatment Plant, Greensboro, N. C.

Imhoff, K. and Fair, G. M. 1956. Sewage Treatment. p. 1-10. John Wiley and Sons, New York.

James, G. V. 1971. A survey of Current Methods of Purifying Domestic Supplies and of Treating Industrial Effluents and Domestic Sewage. Water Treatment. p. 247-281. C.R.C. Press. Cleveland, Ohio.

Kelley, S. M. and Sanderson, W. W. 1958. The Effect of Chlorine in Water on Enteric Viruses. Amer. J. Publ. Hlth. 48:1323-1332.

Kelley, S. M. and Sanderson, W. W. 1960a. Density of Enterovirus in Sewage. J. Water Poll. Control Fed. 32:1269-1273.

Kelley, S. M. and Sanderson, W. W. 1960b. The Effect of Chlorine on Enteric Virus. II. The Effect of Combined Chlorine on Poliomyelitis and Coxsackie Viruses. Amer. J. Publ. Hlth. 50:14-19.

Lothrop, T. L. and Sproul, O. J. 1969. High-level Inactivation of Viruses in Wastewater by Chlorination. J. Water Poll. Control Fed. 41:567-576.

Luftig, R. and Haselkorn, R. 1967. Morphology of a Virus of Blue-green Algae and Properties of its Deoxyribonucleic Acid. J. Virol. 1:344-361.

Luftig, R. and Haselkorn, R. 1968. Studies on the Structure of Blue-green Algal Virus LPP-1. Virology. 34:664-674.

Malina, J. F., Ranganathan, K. R., Moore, B. E. D. and Sagik, B. P. 1974. Poliovirus Inactivation by Activated Sludge. p. 95-107. In J. F. Malina and B. P. Sagik (ed.) Virus Survival in Water and Wastewater Systems. Center for Research in Water Resources, University of Texas, Austin, Texas.

Metcalf, T. G., Wallis, C. and Melnick, J. L. 1974. Virus Enumeration and Public Health Assessments in Polluted Surface Water Contributing to Transmission of Virus in Nature. p. 57-70. In J. F. Malina and B. P. Sagik (ed.) Virus Survival in Water and Wastewater Systems. Center for Research in Water Resources, University of Texas, Austin, Texas.

Miller, A. P. 1962. Water and Man's Health, Washington D. C., Office of Human Resources and Social Development. Agency for International Development. (A. I. D. Community Water Supply, Technical Services., No. 5) In Daniel A. Okun and George Ponquis, Community Wastewater Collection and Disposal. p. 2. World Health Organization, Geneva.

Mosly, J. W. 1967. Transmission of Viral Diseases by Drinking Water. p. 5-23. In Gerald Berg (ed.) Transmission of Viruses by the Water Route. Interscience Publishers, New York.

Nupen, E. M., Bateman, B. W. and McKenny, N. C. 1974. The Reduction of Virus by the Various Unit Processes Used in the Reclamation of Sewage to Potable Water. p. 107-114. In J. F. Malina and B. P. Sagik (ed.) Virus Survival in Water and Wastewater Systems. Center for Research in Water Resources, University of Texas, Austin, Texas.

Rubenstein, S. H., Fenters, J., Orbach, H., Shuber, N., Reed, J. and Malloy, E. 1973. Viruses in Metropolitan Waters: Concentration by Polyelectrolytes, Freeze Concentration, and Ultrafiltration. Jour. Amer. Water Works Assn. 65:200-202.

Safferman, R. S. and Morris, M. E. 1963. Algal Virus: Isolation. Science. 140:679-680.

Safferman, R. S. and Morris, M. E. 1964. Growth Characteristics of the Blue-green Algal Virus LPP-1. J. Bact. 88:771-775.

Safferman, R. S. and Morris, M. E. 1967. Observation on the Occurrence, Distribution, and Seasonal Incidence of Blue-green Algal Viruses. Appl. Microbiol. 15:1219-1222.

Safferman, R. S., Morris, M. E., Sherman, L. A. and Haselkorn, R. 1969. Serological and Electron Microscopic Characterization of a New Group of Blue-green Algal Viruses (LPP-2). Virology 39:775-780.

Safferman, R. S. 1973. Phycoviruses. p. 225-230. In N. G. Carr and B. A. Whitton (ed.) The Biology of Blue-green Algae. University of California Press. LosAngeles, California.

Schneider, I. R., Diener, T. O. and Safferman, R. S. 1964. Blue-green Algal Virus LPP-1: Purification and Partial Characterization. Science. 144:1127-1130.

Shane, M. S., Wilson, S. B. and Fries, C. R. 1967. Virus-host System for use in the Study of Virus Removal. Jour. Amer. Water Works Assn. 59:1184-1186.

Sherman, L. A. and Haselkorn R. 1970a. LPP-1 Infection of the Blue-green Alga Plectonema boryanum. I. Electron Microscopy. J. Virol. 6:820-833.

Sherman, L. A. and Haselkorn R. 1970b. LPP-1 Infection of the Blue-green Alga Plectonema boryanum. II. Viral DNA Synthesis and DNA Breakdown. J. Virol. 6:834-840.

Shuval, H. I., Cymbalista, S., Wachs, A., Zohar, Y. and Goldblum, N. 1966. The Inactivation of Enterovirus in Sewage by Chlorination. p. 37-52. In S. H. Jenkins and L. Mendia (ed.) Advances in Water Pollution Research. Vol. 2. Water Pollution Control Federation, Washington, D. C.

Shuval, H. I., Cymbalista, S., Fatal, B. and Goldblum, N. 1967. Concentration of Enteric Viruses in Water by Hydroextraction and Two-phase Separation. p. 45-56. In Transmission of Viruses by the Water Route. Interscience, New York.

Sorber, C. A., Malina, J. F. and Sagik, B. P. 1971. Monitoring of Low Level Virus in Natural Waters. Applied Micro. 22:334-338.

Smedberg, C. T. and Cannon, R. E. 1976. Cyanophage analysis as a Biological Pollution Indicator-Bacterial and Viral. J. Water Poll. Control Fed. in press.

Smith, K. M., Brown, R. M., Goldstein, D. A. and Walne, P. L. 1966. Culture Methods for the Blue-green Alga Plectonema boryanum and its Virus with an Electron Microscope Study of the Virus Infected Cells. Virology 28:580-591.

Standard Methods for the Examination of Water and Wastewater. 1965. (ed.) Herbert P. Orland, Albany, N. Y.

Stander, G. J. and Van Vuuren, L. R. J. 1969. The Reclamation of Potable Water from Wastewater. J. Water Poll. Control Fed. 41:355-367.

State of North Carolina Department of Natural and Economic Resources. 1975. Regulation Relating to Monitoring and Reporting Wastewater Discharges and Their Effect Upon Receiving Waters as Amended. p. 36. Environmental Management Commission, Raleigh, N. C.

Wallis, C., Melnick, J. L. and Fields, J. E. 1970. Detection of Virus in Large Volumes of Natural Waters by Concentration on Insoluable Polyelectrolytes. Water Research. 4:787-796.

Weidenkopf, S. J. 1958. Inactivation of Type I Poliomyelitis Virus with Chlorine. Virology. 5:56-67.

Zajic, J. E. 1971. Water Pollution Disposal and Reuse. p. 1-29. p. 371-389. Marcel Dekker Inc., New York.

APPENDIX A

SEROLOGICAL SAMPLE SIZE DETERMINATION

The sample size for serological studies was determined according to a binomial distribution equation. A preliminary sample of 20 plaques was serologically typed so to obtain a tentative cyanophage distribution. It was found that LPP-2 predominated over LPP-1 about 9 to 1. These results were used in the following equation:

$f(x) = \binom{n}{x} P^x (1-P)^{n-x}$

$f(x)$ = the probability that the results will be as expected.

n = sample size

x = # of positive results.

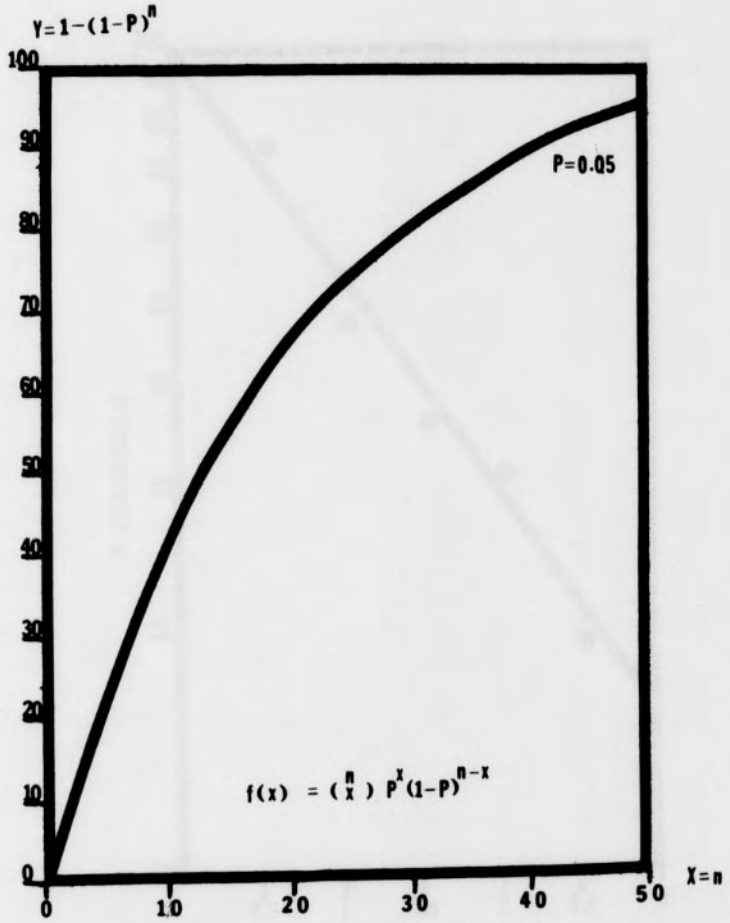
P^x = 90% LPP-2

$(1-P)^{n-x}$ = 10% LPP-1

Different sample sizes were mathematically determined and plotted in association with varying confidence levels (Appendix A_1). The chosen sample size of 48 picked plaques from each sampling date represents a confidence level of 92%.

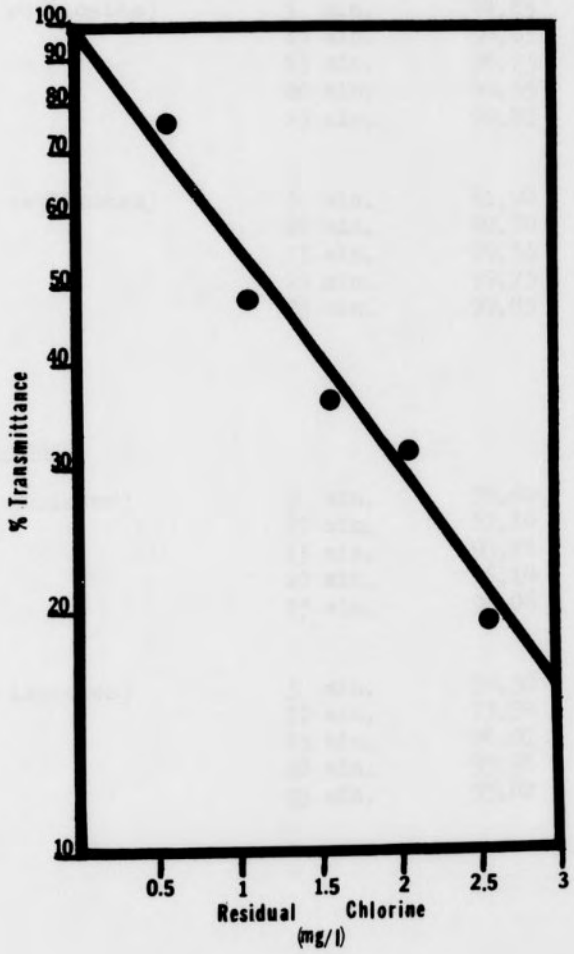
APPENDIX A_1

CONFIDENCE LEVEL DETERMINATION



Appendix B

CHLORINE STANDARDS



APPENDIX C

CYANOPHAGE CHLORINE RESISTANCE: STOCK-ISOLATES

Stock Cyanophage	Contact Time	% Kill	% Survival
1. LPP-1 (2 replicates)	5 min.	44.85	55.15
	10 min.	94.05	5.95
	15 min.	98.75	1.25
	20 min.	99.55	0.45
	25 min.	99.85	0.15
2. LPP-2 (2 replicates)	5 min.	61.90	38.10
	10 min.	92.70	7.30
	15 min.	99.55	0.45
	20 min.	99.75	0.25
	25 min.	99.85	0.15
Wastewater Isolated Cyanophage			
1. LPP-1 (5 isolates)	5 min.	38.60	61.40
	10 min.	67.10	32.90
	15 min.	93.78	6.22
	20 min.	96.14	3.86
	25 min.	98.06	1.94
2. LPP-2 (5 isolates)	5 min.	30.58	69.42
	10 min.	73.34	26.66
	15 min.	90.02	9.98
	20 min.	93.96	6.04
	25 min.	95.82	4.18